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PRODUCTION OF POLYCLONAL ANTIBODIES IN RABBITS

FINAL REPORT

ELIE S. NUWAYSER

SEPTEMBER 30, 1992

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Cholinesterases are widely distributed throughout the body. They hydrolyze acetylcholine (ACh), a compound which serves also as the neurohumoral agent in peripheral junction transmission. Cholinesterases are known to be crucial for cholinergic neurotransmission.

The objective of the Phase I application is to raise antibodies from rabbits against cholinesterase, butyrylcholinesterase, glycosylated butyryicholinesterase, synthetic peptides that mimic selected areas of the HIV virus proteins, and also against monoclonal antibodies which inhibit the catalytic activity of cholesterase. The immunogens used in the preparation of the antibodies will be supplied by WRAIR.

During Phase I of the SBIR contract polyclonal antibodies were successfully raised from rabbits against acetylcholinesterase, butrylcholinesterase, and five synthetic peptides using immunogens supplied by WRAIR. The binding capacity of the peptide antisera was determined by enzyme linked immunoassay. Most of the titers at half maximal absorbance were greater than 1×10^4. All antibodies were submitted to WRAIR. Antibodies against the enzymes were assayed by WRAIR for IgG and enzyme content.

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I. IDENTIFICATION OF THE OPPORTUNITY

Cholinesterases are widely distributed throughout the body. They hydrolyze acetylcholine (ACh), a compound which serves also as the neurohumoral agent in peripheral junction transmission. In addition to neurons, they are also found in plasma, brain, muscle, and other tissues. The cholinesterases fall into two separate families: true cholinesterase, also known as acetylcholinesterase (AChE); and pseudocholinesterase, also known as butyrylcholinesterase (BuChE). Cholinesterase occurs in a variety of molecular forms. It is crucial for cholinergic neurotransmission and occurs at high concentrations in the immediate vicinity of nerve endings where it hydrolyzes ACh in less than a millisecond (**Goodman and Gilman, 1990**). Butyrylcholinesterase is present to a limited extent in neural elements and is also present in plasma, liver, pancreas and other organs.

The function of AChE is to terminate the action of ACh at the junctions of the various cholinergic nerve endings with their effecter organs or postsynaptic sites. Drugs that inhibit AChE are called anticholinesterase (anti-ChE). They cause accumulation of ACh at cholinergic receptor sites and produce effects equivalent to excessive stimulation of these receptors throughout the peripheral and central nervous systems. This property has led to their use as chemical warfare agents, as agricultural insecticides and as toxic agents.

The objective of the Phase I application is to raise antibodies from rabbits against cholinesterase, butyrylcholinesterase, glycosylated butyrylcholinesterase, synthetic peptides that mimic selected areas of the HIV virus proteins, and also against monoclonal antibodies which inhibit the catalytic activity of cholesterase. The immunogens used in the preparation of the antibodies will be supplied by WRAIR. The binding capacity of the antibodies to the immunogen will be determined by enzyme linked immunosorbant assay (ELISA). The antibodies will be submitted to WRAIR for use in the development of assay procedures, localization of cholinesterase activity, and understanding the mechanisms of a action in various tissues. Antibodies prepared against peptides that mimic selected areas of the HIV virus proteins will be used to identify the virus, localize it, and for elucidating the mode of action of new treatment modalities.

II. SIGNIFICANCE

In view of the widespread distribution of cholinergic neurons, anti-ChE agents have received extensive application as potential chemical-warfare nerve gases. Initial work prior to 1940 with anti-ChE focussed on "reversible" agents, of which physostigmine is the best example. Since then several extremely toxic organophosphorus nerve gases were synthesized which "irreversibly" inactivate AChE. Examples of these include sarin, soman, and tabun. Other compounds are employed as agricultural insecticides, the foremost examples being parathion, malathion, and diazinon.

Immunochemical methods offer an important tool for understanding the cholinesterases and anticholinesterases. They can be used to determine the source, and measure tissue levels of the enzyme protein, to localize inactive or other forms of the enzyme and to understand the structural and functional significance of cholinesterase turnover, fate and polymorphism. Antibodies raised against cholinesterase may be used to monitor binding and or inhibition of mouse monoclonal antibodies and, in the case of anti-idiotypic antibodies, as probes for elucidating the topography of

the original antigen. Because of their high sensitivity, selectivity, and specificity, immunochemical methods are important for elucidating the mode of action of nerve gases.

III. PHASE I RESULTS

Seven antisera were prepared during Phase I. The antisera were raised in SPF New Zealand White rabbits against the following:

1. Horse serum butyrylcholinesterase
2. Bovine fetal serum acetylcholinesterase
3. Five synthetic peptides labeled A through E and prepared by WRAIR to represent amino acid sequences selected from the area surrounding the gorge of the active site of bovine fetal cholinesterase.

The binding capacity of the peptide antisera was determined by enzyme linked immunoassay. Most of the titers at half maximal absorbance were greater than 1×10^4. All antibodies were submitted to WRAIR. Antibodies against the enzymes were assayed by WRAIR for IgG and enzyme content.

A description of the protocols used to prepare these antibodies is given below:

A. Horse Serum Butyrylcholinesterase Enzyme

A 4.5 ml suspension of horse serum butyrylcholinesterase enzyme in 50% glycerol was received from the contracting officer's representative (COR) at Walter Reed Army Institute of Research. The enzyme activity was approximately 7,000 U/ml. The enzyme was stored at -20°C in a non-defrosting freezer. The use of 50% glycerol serves several purposes: 1) it prevents the growth of bacteria, 2) it stabilizes the highly purified enzyme and 3) it prevents the formation of ice crystals at -20°C.

1. Immunization

For injection, 60 µl of enzyme (420 units) was diluted in 0.5 ml of sterile normal saline immediately before injection. No adjuvant was used. The initial injections of enzyme were administered IM to five rabbits on April 20, 1992 using the experimental protocol received from COR (see below). The mean weight of the rabbits was 2.84 kg $\pm$ 0.21 s.d. The weight history of the rabbits is given in **Table 1**. They were numbered 621, 622, 623, 625, and 626.

2. Blood Collection

The procedure used for blood collection is as follows:

The rabbit was placed in a restrainer and was injected with a dose of 0.5-1 mg/kg SC of acepromazine as a tranquilizer and to dilate blood vessels. The ear artery was dilated by finger tapping and then the 23 gauge needle of a 12" IV infusion set was inserted into the artery and a calibrated 250 µl Microcaps Nelson capillary tube was attached to the other end. Blood was drawn into the capillary tube and immediately mixed with 2.25 ml of distilled water in a 5 ml screw-cap polyethylene vial. The vials were labeled according to the system suggested by COR as follows:

BChE-1 - Rabbit Number - Sample No. - Date

The hemolyzed blood samples were immediately frozen until shipped. The rabbits were healthy after injection and did not show any adverse side effects.

3. Immunization and Blood Collection Protocol

The immunization and blood collection protocol for the antibodies against horse serum butyrylcholinesterase is shown in Table 2. The protocol was developed by COR specifically for the enzyme. The table shows the day and date of initial immunization, the booster injection, bleeds, and shipment of the blood to WRAIR.

4. Results

The rabbits remained healthy throughout the study period. All rabbits gained weight (Table 1). Enzyme activity and IgG content of the antisera was determined by WRAIR. Figure 1 shows the mean level of the enzyme and IgG. The data was provided courtesy of the Contracting Officer's Representative, Mrs. Mary K. Gentry, and shows good response to the immunization protocol devised by the COR.

B. Fetal Bovine Serum Acetylcholinesterase

Approximately 8 ml of fetal bovine serum acetylcholinesterase suspended in 10% glycerol was received from WRAIR. The enzyme activity was approximately 7,300 U/ml. Upon receipt, the enzyme was stored at -4°C, since it is not stable when frozen and thawed, but can be stored at this temperature without freezing because of the 10% glycerol, which also prevents the growth of microorganisms.

1. Immunization

For injection, 300 μl (2000 units) of the enzyme suspension in glycerol was diluted in 0.5 ml of sterile normal saline. The dilution and mixing was performed immediately. No adjuvant was used. The initial injection was administered IM to 5 rabbits on May 11, 1992 using the experimental protocol received from COR (see below). The rabbits received 300 μl of the enzyme preparation at each period except injection No. 5 where each rabbit received approximately 10 μl because of losses incurred during the previous four injections. The mean weight of the five rabbits was 2.87 kg $\pm$ 0.31 s.d. The weight history of the rabbits is given in Table 1. They were numbered 627, 629, 630, 631, and 632.

2. Blood Collection

The procedure for blood collection, dilution and storage is the same as the procedure used for horse serum butyrylcholinesterase (see previous section).

3. Immunization and Blood Collection Protocol

The immunization and blood collection protocol for the antibodies against fetal serum acetylcholinesterase is shown in Table 3. The protocol was developed by COR specifically for the enzyme. The table shows the day and date of the initial immunization, the booster injections, bleeds, and shipment of the blood to WRAIR.

4. Results

The rabbits were healthy throughout the immunization and blood collection protocol. All rabbits gained weight. Enzyme activity and IgG content of the antisera was provided courtesy of Mrs. Mary K. Gentry, the Contracting Officer's Representative. The mean values for the enzyme and IgG for the five rabbits is presented in **Figure 2**, and shows good response to the immunization protocol devised by the COR.

C. Synthetic Peptides from Fetal Bovine Serum Acetylcholinesterase

Five liposome-encapsulated peptide mixtures (cocktails) labeled A, B, C, D, and E and five non-encapsulated peptide mixtures for use in enzyme linked immunosorbant assay (ELISA) were received from WRAIR.

According to the COR, the peptide cocktails were prepared by WRAIR and are composed of varying mixtures of four synthetic peptides which were selected from the area surrounding the gorge of the active site of fetal bovine serum acetylcholinesterase. The four synthetic peptides represent the following amino acid sequence of the enzyme:

Peptide	Amino Acid Sequence	Number of Amino Acids
1	49-91	43
2	264-299	36
3	321-362	42
4	420-454	35

The five cocktails injected into rabbits were prepared from the synthetic peptides according to the following scheme:

Cocktail	Peptides Contained
A	1,2,3
B	1,2,4
C	1,3,4
D	2,3,4
E	1,2,3,4

The ELISA antigens were suspended in phosphate buffered saline with 0.1% sodium azide and 10% DMSO. Each preparation was assumed to be 1 mg/ml of peptide.

1. Immunization

One and a half milliliter (1.5 ml) of each peptide was emulsified with 1.5 ml alum suspension (supplied by WRAIR) using a luer one-way stopcock and were injected immediately after emulsification.

Each peptide was administered to two rabbits by injecting 0.75 ml IM into the left thigh and 0.75 ml IM into the right thigh for a total of 1.5 ml per rabbit. The initial injections were given on

May 11, 1992. The boost vaccines were administered similarly, except each rabbit received 0.5 ml in each thigh (total of 1 ml) due to shortage of vaccine because of syringe losses from previous injections. The mean weight of the 10 rabbits was 2.46 kg $\pm$ 0.08 s.d. The weight history of the rabbits is given in Table 1. The rabbits were numbered as follows:

Peptide Cocktail	Rabbit Numbers
A	633, 634
B	635, 636
C	637, 638
D	639, 640
E	641, 642
Repeat of D	693, 694

Peptide D was repeated in two additional rabbits on July 15, 1992 because the ELISA results were not promising (see below).

2. Blood Collection

The rabbit was placed in a restrainer and was injected with a dose of 0.5-1 mg/kg SC of acepromazine as a tranquilizer and for dilating the blood vessels. The ear artery was dilated by finger tapping and a 23 gauge needle of a 12" infusion set was inserted into the artery and 10 ml of blood was drawn. The blood samples were allowed to sit at room temperature for 1 hour and then in the refrigerator at 4°C overnight. The sera were separated by centrifugation at 2,000 rpm for 15 minutes and then frozen until shipment to WRAIR.

3. Immunization and Blood Collection Protocol

The immunization and blood collection schedule for the peptide antibodies is shown in Table 4. The table shows the day and date of the initial immunization, the booster injections, test bleeds, production bleeds, and shipping of the sera to WRAIR.

4. Results

A discussion of the ELISA results for the five peptide cocktails is presented in the next section.

D. Monitoring Antibody Production by ELISA

1. Approach

Enzyme linked immunosorbant assays (ELISA) was performed on rabbit plasma prepared from bleeds taken prior to the initial injection of the peptides to establish non-specific binding levels, and from bleeds taken after each booster injection to monitor the buildup of antisera. The ELISA binding assays were performed in a "sandwich" format in 96 well microtiter plates.

Briefly, the peptides provided by Walter Reed Institute of Research (WRAIR) for the elicitation of antibodies were coated to the inner surface of microtiter wells where they act as antigens to the elicited antibodies. Serial dilutions of the rabbit plasma were made in the microtiter plates to allow antibodies present in the plasma to bind to the coated antigens. The captured

antibodies were then reacted with enzyme-labeled antibodies raised against rabbit IgG's. Colorimetric substrates developed by the conjugated enzymes were detected spectrophotometrically in a microplate reader. The optical density measurements were transmitted on line to a computer for data storage and analysis of antibody titers.

2. Choice of Microtiter Plates

Initial studies focussed on an evaluation of passive adsorption versus covalent coupling of peptide to microtiter plates, and the optimization of an ELISA assay to use as a positive control for the antisera screening assays.

Various concentrations of human Angiotensin II peptide, with the sequence Asp-Arg-Val-Tyr-Lle-His-Pro-Phe, (Peninsula) were immobilized in both covalent and non-covalent polystyrene microtiter plates (Costar). The immobilized peptides were detected using a 1: 4,000 dilution of rabbit anti-Angiotensin II antiserum (Peninsula), goat-anti-rabbit-HRP (Pierce), and ABTS (Sigma). The results from one of the comparisons is shown in **Figure 3**. These data indicate that microtiter plates activated with covalent coupling reagents provide superior assay sensitivity, and suggest the use of 10 μg/ml peptide for optimal coupling.

Angiotensin II (10 μg/ml) was next immobilized to microtiter plates and detected with a range of dilutions of the anti-Angiotensin II antiserum. As shown in **Figure 4**, the titer of the antiserum approached the 1: 90,000 stated by Peninsula Labs. These data indicate that close to optimal concentrations of the various immunoreagents and assay conditions were employed in the covalently activated plate. So long as the peptides to be provided by WRAIR are immobilized to the microtiter plates as efficiently as Angiotensin II, the assay will provide sufficient detection sensitivity for screening the upcoming antisera.

3. Optimization of the ELISA Conditions

Peptides A, B, C, D, and E were immobilized to microtiter plates using both passive adsorption and covalent coupling procedures. To determine optimal conditions for passive adsorption, various pH values and ionic strengths of the adsorption solutions were examined. Covalent coupling was performed on NH_2-plates (Costar) using the homobifunctional coupling reagent Bis (sulfosuccinimidyll) suberate. Angiotensin II and IgG were used as controls. The method of **Griesmann, et. al. (1991)**, was used to assess the effectiveness of the immobilization procedures. In this procedure, free amino groups of the immobilized peptide are biotinylated and detected with streptavidin-peroxidase. Where applicable, this procedure can be used to demonstrate the presence of peptides in the microtiter plates in the event of a negative antisera screening result.

Passive adsorption to polystyrene microtiter plates was demonstrated by Griesmann's procedure for all of the peptides. We confirmed the report by **Geerlings, et. al. (1988)**, that the effects of pH and salt concentration on the efficiency of adsorption of peptides to polystyrene are rather complex. Peptides A, B, and C were all absorbed efficiently at 50 μg/ml in 0.2 M salt at pH 4.0 **(Figure 5)**. Only Peptide C was absorbed efficiently in 0.2 M salt at pH 9.2 **(Figure 6)**. Peptide A absorbed most efficiently at pH 4.0, but also absorbed efficiently at pH 9.2. No pH dependence was observed for peptides D and E. The effects of salt concentration on the adsorption of the different peptides were quite varied. Initial data showed increases, decreases, and no change in adsorption of the different peptides as the salt concentration was increased from 0.2 M to 0.6 M.

As reported for Angiotensin II (See above) covalent coupling required lower concentrations of peptide than passive adsorption. Covalent coupling to NH_2 plates was demonstrated by Gries-

mann's procedure for peptides A, B, and E, but not for peptides C and D (Figure 7). Lack of a positive reaction in the Griesmann procedure does not necessarily indicate non-coupling of the peptide to the plate. If a peptide lacks lysine residues it cannot be detected by this procedure since its free amino group is utilized by the homobifunctional coupling reagent. Covalent coupling of Angiotensin II, which lacks lysine, to NH_2 plates was confirmed using rabbit anti-Angiotensin II antiserum (Peninsula), but not by the Griesmann procedure. If peptides C and D contain lysine residues, the data probably indicates inefficient coupling using this procedure.

4. Methods and Procedures

The following are the protocols used to determine the titers of antisera in rabbits immunized with peptides A, B, C, D, and E supplied by WRAIR.

a. Covalent Coupling

All coupling procedures are performed on NH_2 plates (Costar).

1. Prepare a 1 mg/ml solution of Bis (sulfosuccinimidyl) suberate (Pierce) in phosphate buffered saline (PBS, pH 7.4, 0.15M NaCl). Add 100 µl to each microwell and let react for 30 minutes at room temperature.

2. Aspirate and wash three times with PBS.

3. Prepare peptide dilutions of 10 µg/ml in PBS and add 100 µl to each microwell. Complete the coupling procedure for one hour at room temperature with shaking.

4. Aspirate and wash three times with PBS.

5. Begin assay procedure with Step 1.

b. Passive Adsorption

All adsorption procedures are performed on High Bind plates (Costar)

1. Prepare 50 µg/ml of each peptide in sodium phosphate, pH 4.0, 0.2M NaCl. Add 100 µl of peptide solution to each microwell.

2. Passively adsorb overnight at 4°C.

3. Aspirate and wash three times with PBS.

c. Control Wells

Immobilize human Angiotensin II peptide (Peninsula) to the microwells using the above procedure. A 1:4,000 dilution of rabbit anti-Angiotensin II (Peninsula) is used for detection.

d. Assay Procedure

1. Prepare a blocking solution of 3% BSA (Armour Pharmaceuticals) in PBS. Add 200 µl to each microwell and block for one hour at room temperature with shaking.

2. While blocking, prepare stocks of anti-sera dilutions at 1:100 in PBS-BSA.

3. Aspirate and add 150 µl of PBS-BSA to all microwells. To each well of the first row of the plate (Row A), pipette 50 µl of the appropriate 1:100 anti-sera dilution. Working down the plate, pipette 50 µl of anti-sera solution from wells in Row A to wells in Row B pipetting up and down three times after each addition to thoroughly mix the solution. Repeat this procedure sequentially down the plate preparing 1/4 anti-sera dilutions. Discard the remaining 50 µl from Row H of the final dilution.

4. Peptide/anti-sera incubation can be completed in 2-3 hours at room temperature or overnight at 4°C.

c. Colorimetric Immunodetection Procedure

1. Aspirate the anti-sera solutions from each well and wash all wells three times with PBS-0.05% Tween-20.

2. Prepare the enzyme reagent, goat anti-rabbit horseradish peroxidase (Pierce), at a 1:1,000 dilution in PBS-BSA. Add 100 µl to each microwell and incubate for one hour at room temperature with shaking.

3. Aspirate and wash three times with PBS-Tween.

4. Prepare a 1 mg/ml dilution of the immunodetection reagent ABTS (2,2'-Azino-bis(3-Ethylbenz-Thiazoline-6-Sulfonic Acid), in phosphate-citrate buffer, pH 5.0. Wait until dissolved and add 0.01% Tween-20. Immediately prior to detection add 1 µl/ml H_2O_2 to the substrate solution. For detection procedures, pipette 100 µl of substrate solutions in each microwell.

5. Shake at room temperature and read at 405 nm every 10 minutes until the wells reach maximum absorbance.

5. Antibody Titer of Plasma Drawn Thirty Days after Immunization

Peptides A, B, C, D, and E were immobilized to microtiter plates using optimal passive adsorption and covalent coupling procedures determined in the assay optimization study reported above. Initial screening using covalent plates indicated that the antisera should be diluted to 1/400, then to dilutions below 10^6 in order to determine antibody titers. Two sets of antisera were diluted from 1/400, following in four fold steps to 1/6,553,600. The antisera were then screened on both covalent and passively adsorbed microtiter plates.

In this study, controls of pre-injection plasma were run in parallel for each rabbit. The control values were subtracted from the values obtained for the plasma sample collected 30 days after immunization. The titration curves for the peptides covalently coupled to polystyrene microtiter plates are shown in Figures 8 through 12. The titration curves for the peptides passively immobilized on polystyrene microtiter plates are shown in Figures 13 through 17. A summary of the titers at half maximal absorbance which were determined from the above curves is presented in Table 5. It should be noted that rabbit no. 640 caught her leg in the cage and broke it. She was bled only one day after booster injection, compared to nine days for the other rabbits.

IV. REFERENCES

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2. Geerlings, et al., J. Immunol. Methods, 106, 239 (1988).
3. Griesmann, et al., J. Immunol. Methods, 138, 25 (1991).

TABLE 1

RABBIT WEIGHTS AS OF (AUGUST 27, 1992)

SAMPLE	RABBIT NO.	WEIGHT (kg) April 14, 1992	WEIGHT (kg) April 20, 1992	WEIGHT (kg) April 29, 1992	WEIGHT (kg) June 2, 1992	WEIGHT (kg) June 29, 1992	WEIGHT (kg) July 2, 1992	WEIGHT (kg) July 29, 1992	WEIGHT (kg) August 27, 1992
BChE-1	621	2.15	2.37	2.63	3.30	3.65		3.95	4.10
BChE-1	622	2.25	2.45	2.61	3.00	3.37		3.75	3.85
BChE-1	623	2.39	2.65	3.00	3.80	4.25		4.65	4.70
BChE-1	625	2.50	2.88	3.05	3.55	3.95		4.05	3.90 *
BChE-1	626	2.47	2.73	2.90	3.55	3.98		4.25	4.30
AChE-1	627	2.55	2.83	3.25	3.95	4.55		4.95	4.70 *
AChE-1	629	2.12	2.35	2.65	3.20	3.72		4.15	4.20
AChE-1	630	2.53	2.85	3.15	3.42	4.24		4.55	4.55
AChE-1	631			2.63	3.30	3.82		4.20	4.20
AChE-1	632			2.65	3.50	4.00		4.35	4.25 *
PEPTIDE A	633			2.35	3.25	3.90		4.25	4.25
PEPTIDE A	634			2.60	3.15	3.80		4.15	4.10 *
PEPTIDE B	635			2.43	3.40	4.05		4.50	4.55
PEPTIDE B	636			2.55	3.40	3.85		4.35	4.30 *
PEPTIDE C	637			2.45	3.15	3.68		4.20	4.35
PEPTIDE C	638			2.45	3.30	3.75		4.00	3.80 *
PEPTIDE D	603						2.35	3.30	3.75
PEPTIDE D	604						2.33	3.20	3.65
PEPTIDE E	641			2.52	3.25	4.10		4.45	4.60
PEPTIDE E	642			2.45	3.05	3.40		3.85	3.75 *
PEPTIDE D+	639			2.40	3.10	3.55		4.00	4.00
PEPTIDE D+	640			2.35	2.75				

* = Lost weight compared to the previous week
\+ = Discontinued

Note: Production bleeds on peptides and test bleeds on AChE-1 and BChE-1 were done on previous days, (8/26/92).

TABLE 2

EXPERIMENTAL PROTOCOL FOR ANTIBODY PRODUCTION AGAINST

HORSE SERUM BUTYRYLCHOLINESTERASE IN RABBITS

ANIMAL NOS. 621, 622, 623, 625, AND 626

DAY	DATE	BChE-1	ACTIVITY	DATE SHIPPED
1	April 20, 1992	B-0, I-1	Init. Control Bleed	May 6, 1992
2	April 21, 1992	B-1	Bleed	May 6, 1992
4	April 23, 1992	I-2	Boost	N/A
5	April 24, 1992	B-2	Bleed	May 6, 1992
8	April 27, 1992	I-3	Boost	N/A
9	April 28, 1992	B-3	Bleed	May 6, 1992
11	April 30, 1992	I-4	Boost	N/A
12	May 1, 1992	B-4	Bleed	May 6, 1992
15	May 4, 1992	I-5	Boost	N/A
16	May 5, 1992	B-5	Bleed	May 6, 1992
39	May 28, 1992	B-6	Bleed	June 1, 1992
53	June 11, 1992	B-7	Bleed	June 17, 1992
67	June 25, 1992	B-8	Bleed	June 25, 1992
71	June 29, 1992	I-6	Boost	N/A
73	July 1, 1992	B-9	Bleed	July 6, 1992
74	July 2, 1992	B-10	Bleed	July 6, 1992
81	July 9, 1992	B-11*	Bleed	July 13, 1992
95	July 23, 1992	B-12	Bleed	July 27, 1992
109	August 6, 1992	B-13	Bleed	August 10, 1992
129	August 26, 1992	B-14	Bleed	September 1, 1992
137	September 3, 1992	B-15	Bleed	September 8, 1992
148	September 14, 1992	I-7	Boost	N/A
149	September 15, 1992	B-16	Bleed	September 21, 1992
150	September 16, 1992	B-17	Bleed	September 21, 1992
151	September 17, 1992	B-18	Bleed	September 21, 1992
152	September 18, 1992	B-19	Bleed	September 21, 1992
159	September 25, 1992	B-20	Bleed	September 29, 1992
159	September 25, 1992	Ex	Exsanguinate	September 29, 1992

* - 7/15/92 re-bleed for No. 626 (B-11 spilled) - Day 87

TABLE 3

EXPERIMENTAL PROTOCOL FOR ANTIBODY PRODUCTION AGAINST

FETAL BOVINE SERUM ACETYLCHOLINESTERASE IN RABBITS

ANIMAL NOS. 627, 629, 630, 631, AND 632

DAY	DATE	AChE-1	ACTIVITY	DATE SHIPPED
1	May 11, 1992	B-1, I-1	Init, Control Bleed	June 1, 1992
2	May 12, 1992	B-1	Bleed	June 1, 1992
4	May 14, 1992	I-2	Boost	N/A
5	May 15, 1992	B-2	Bleed	June 1, 1992
8	May 18, 1992	I-3	Boost	N/A
9	May 19, 1992	B-3	Bleed	June 1, 1992
11	May 21, 1992	I-4	Boost	N/A
12	May 22, 1992	B-4	Bleed	June 1, 1992
(15)	May 26, 1992	I-5	Boost	N/A
(17)	May 27, 1992	B-5	Bleed	June 1, 1992
(40)	June 19, 1992	B-6	Bleed	June 22, 1992
53	July 2, 1992	B-7	Bleed	July 6, 1992
67	July 16, 1992	B-8	Bleed	July 27, 1992
71	July 20, 1992	B-9	Bleed	July 27, 1992
72	July 21, 1992	I-6	Boost	N/A
73	July 22, 1992	B-10	Bleed	July 27, 1992
74	July 23, 1992	B-11	Bleed	July 27, 1992
82	July 31, 1992	B-12	Bleed	August 10, 1992
86	August 6, 1992	B-13	Bleed	August 10, 1992
108	August 26, 1992	B-14	Bleed	September 1, 1992
116	September 3, 1992	B-15	Bleed	September 8, 1992
129	September 16, 1992	B-16	Bleed	September 21, 1992
138	September 25, 1992	B-17	Bleed	September 29, 1992
138	September 25, 1992	Ex	Exsanguinate	September 29, 1992

TABLE 4A

EXPERIMENTAL PROTOCOL FOR ANTIBODY PRODUCTION FOR PEPTIDES

ANIMAL NOS. 633, 634, 635, 636, 637, 638, 641, AND 642

DAY	DATE	PEPTIDES A, B, C, E,	ACTIVITY	DATE SHIPPED
1	May 11, 1992	B–0, I–1	Init, Control Bleed	N/A
22	June 1, 1992	I–2	Boost	N/A
31	June 10, 1992	B–1	Test Bleed	N/A
59	July 8, 1992	B–2	Production Bleed	July 13, 1992
73	July 22, 1992	B–3	Production Bleed	July 27, 1992
87	August 5, 1992	B–4	Production Bleed	August 10, 1992
108	August 26, 1992	B–5	Production Bleed	September 1, 1992
122	September 9, 1992	B–6	Production Bleed	September 14, 1992
136	September 23, 1992	B–7 – Ex	Exsanguinate	September 29, 1992

TABLE 4B

ANIMAL NOS. 693 AND 694

DAY	DATE	PEPTIDE D–2	ACTIVITY	DATE SHIPPED
1	July 15, 1992	B–0, I–1	Control Bleed, Initial	N/A
59	September 11, 1992	I–2	Boost	N/A
71	September 23, 1992	B–1 – Ex	Exsanguinate	September 29, 1992

137,921784

TABLE 5

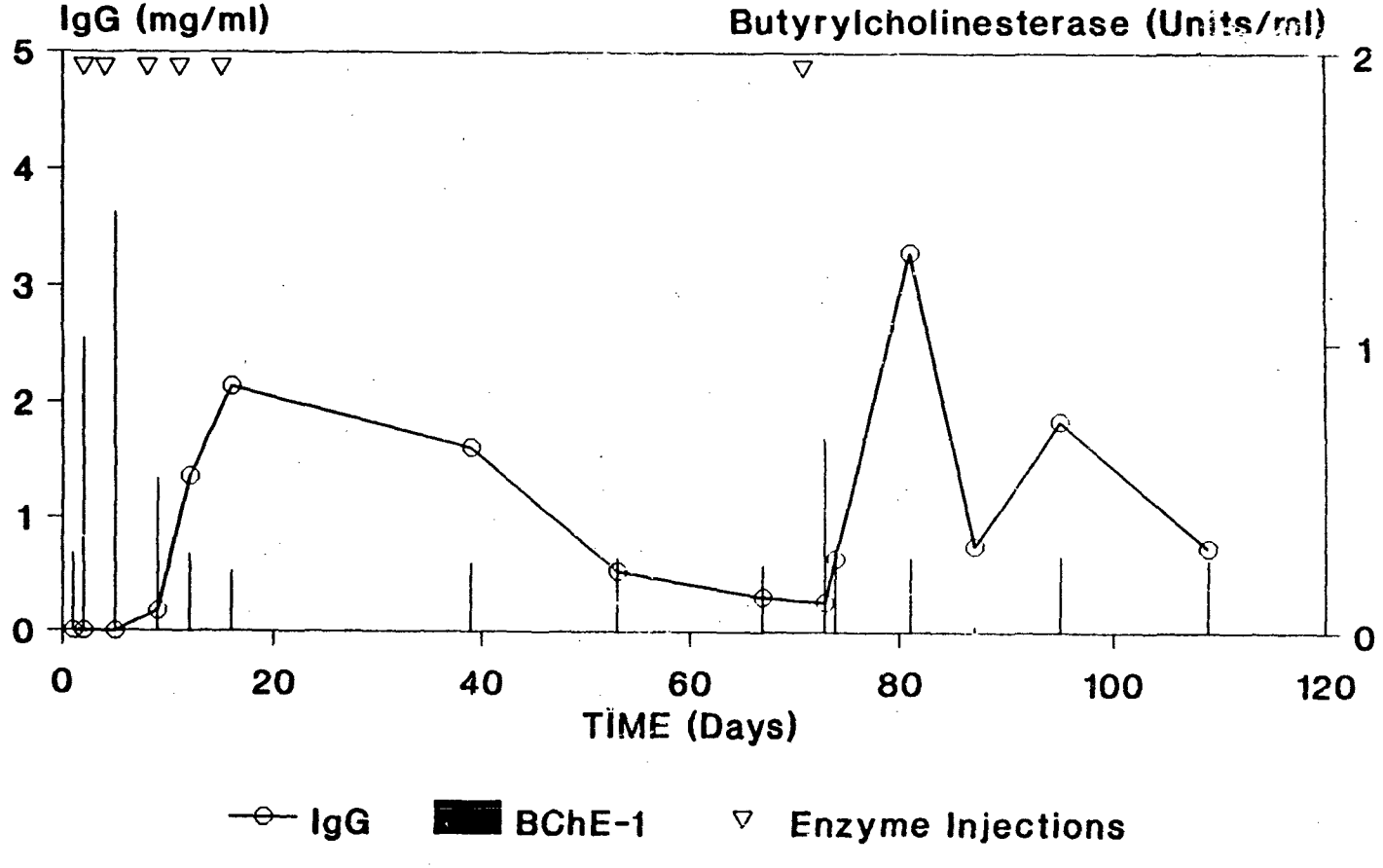
TITER AT HALF MAXIMAL ABSORBANCE OF PEPTIDES A, B, C, D, & E

PEPTIDE	RABBIT	TITER	
		COVALENT PLATES	PASSIVE PLATES
A	633	2.0×10^4	2.0×10^4
A	634	8.0×10^4	4.5×10^4
B	635	1.5×10^4	1.0×10^4
B	636	8.0×10^4	6.5×10^4
C	637	3.0×10^5	1.7×10^5
C	638	3.0×10^4	1.7×10^4
D	639	3.5×10^2	8.0×10^2
D	640	0	0
E	641	4.5×10^4	4.0×10^4
E	642	5.0×10^4	4.0×10^4

137,9217tb5

FIGURE 1

Plasma Level of Horse Serum Butyrylcholinesterase and IgG in Rabbit Antiserum (n=5)

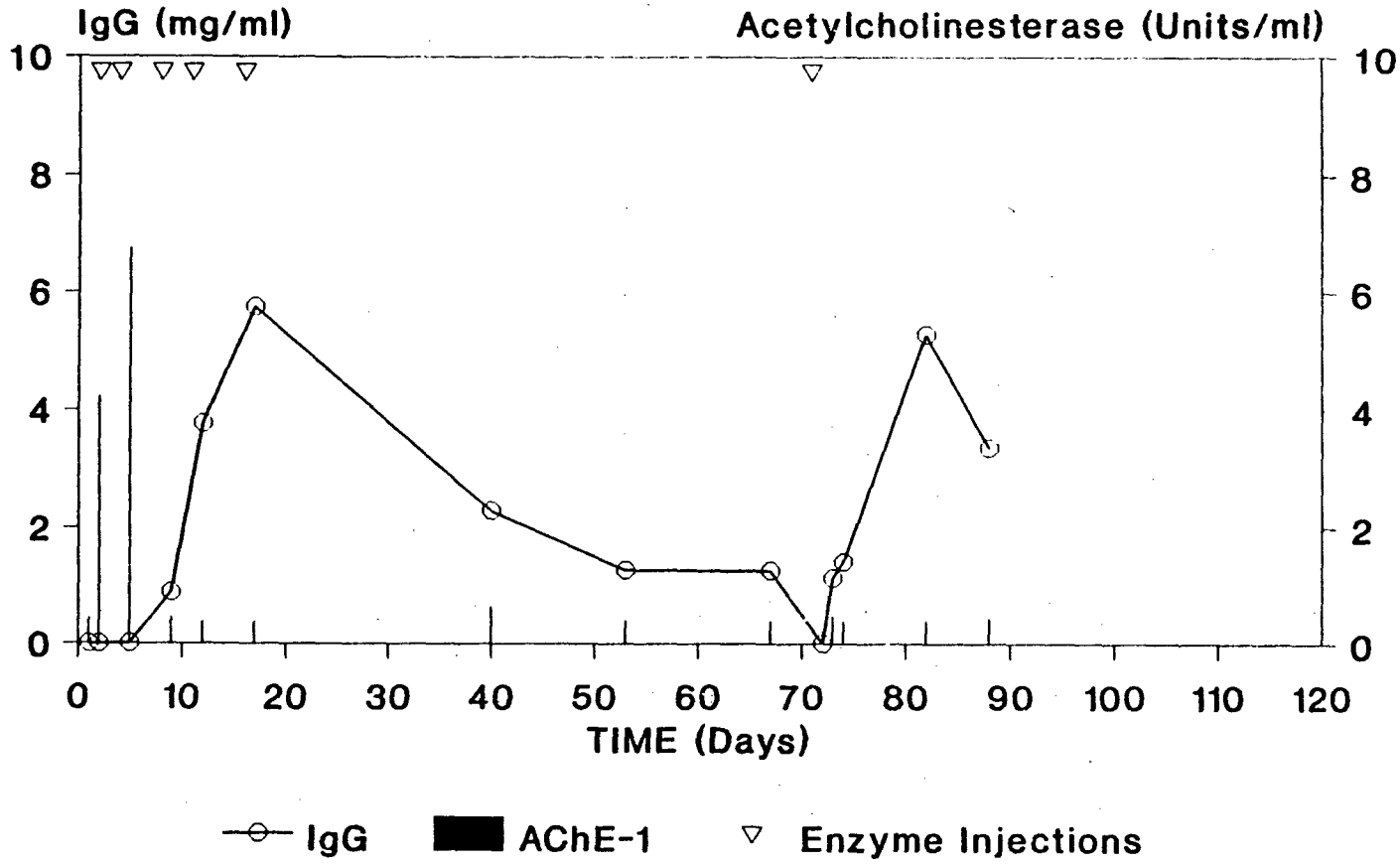


Data courtesy of Mrs. M.K. Gentry, WRAIR

603#BChE-1

FIGURE 2

Plasma Level of Fetal Bovine Serum Acetylcholinesterase and IgG in Rabbit Antiserum (n=5)

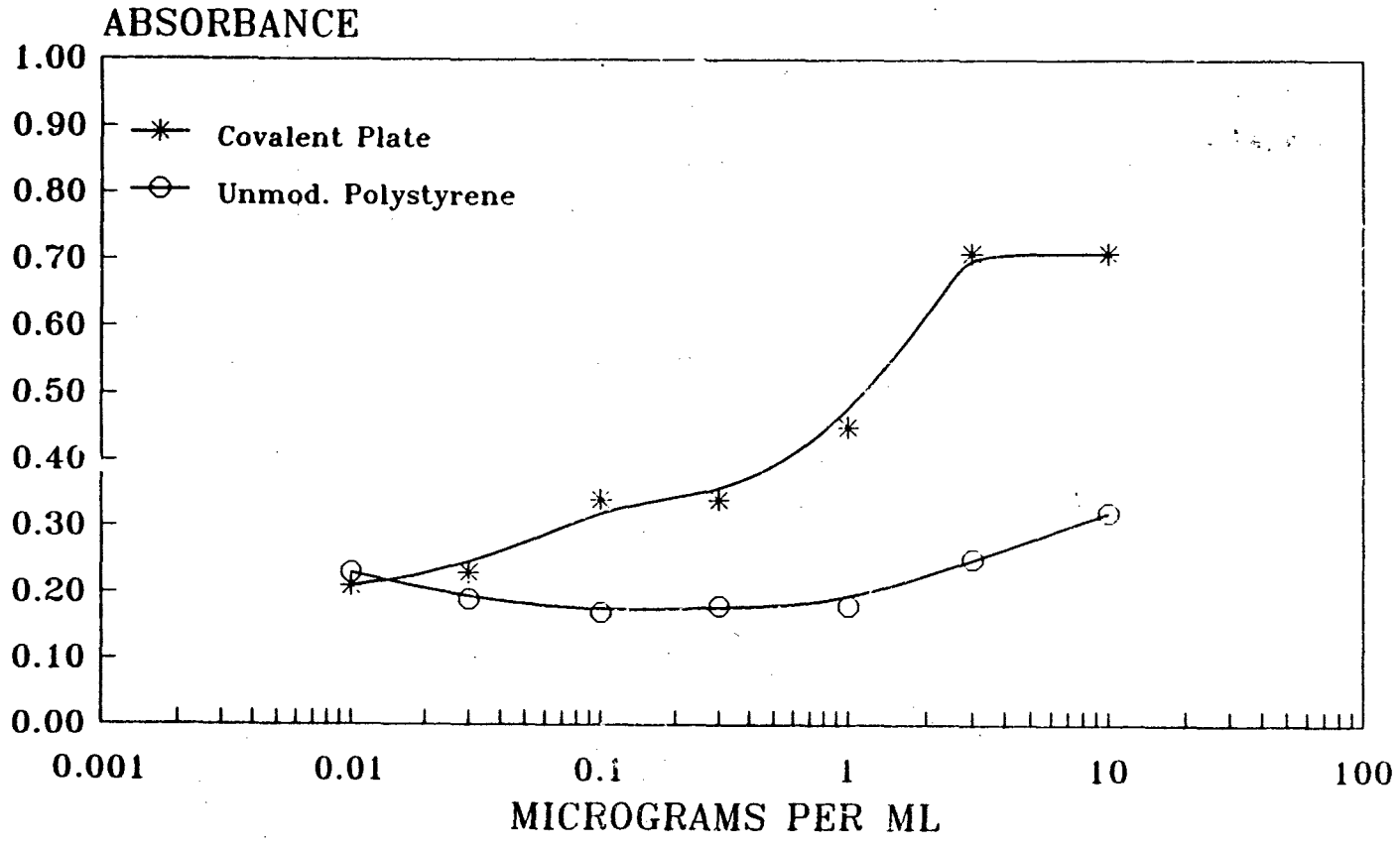


Data courtesy Mrs. M.K. Gentry, WRAIR

603#AChE-1

FIGURE 3

ELISA's for Angiotensin II Performed on Covalent and Unmodified Polystyrene Microtiter Plates *

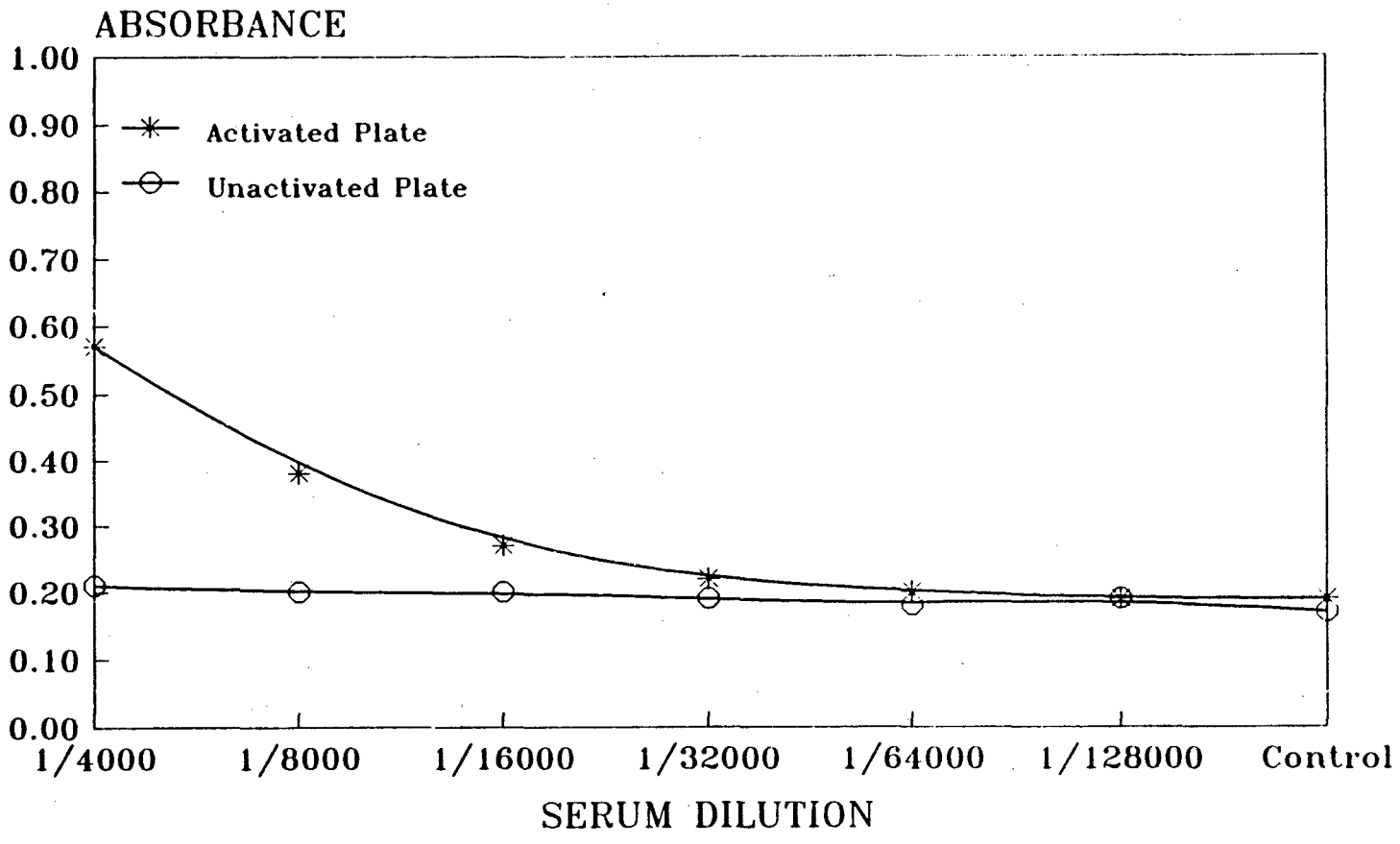


3#BMA-01

* @ 1:4,000 Serum dilution

FIGURE 4

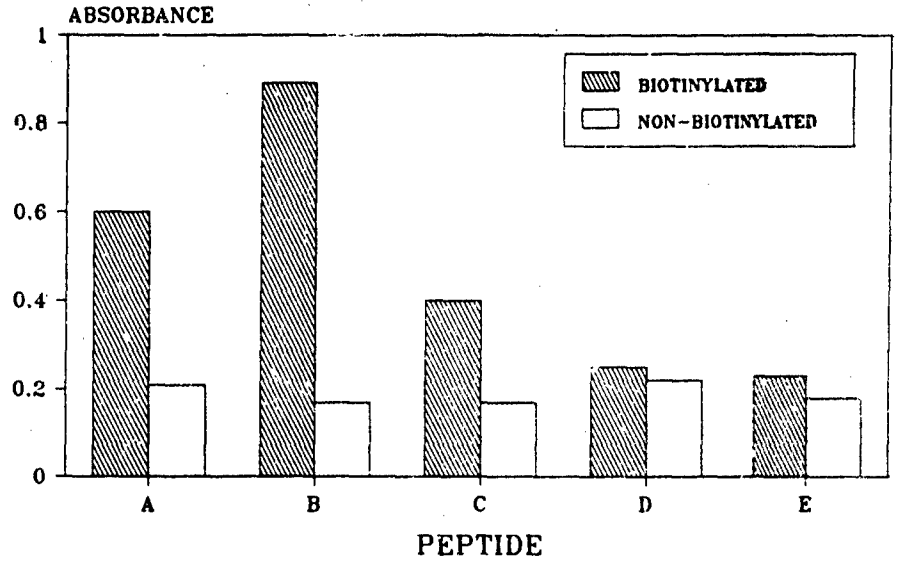
ELISA's for Anti-Angiotensin II Performed on Activated and Unactivated Covalent Plate



3#BMA-02

FIGURE 5

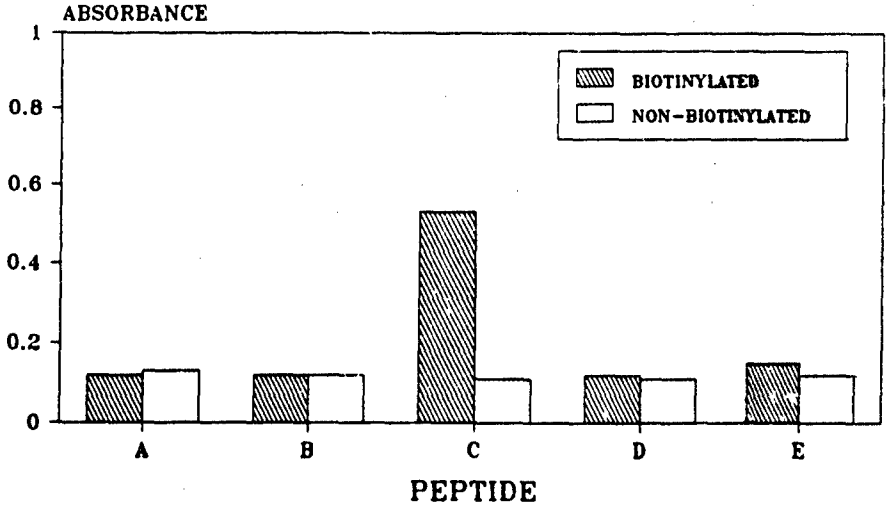
Passive Adsorption of Peptides to
Microtiter Plates
at pH 4.0



3#BMA-07

FIGURE 6

Passive Adsorption of Peptides to
Microtiter Plates
at pH 9.2



3#BMA-08

3#COMB11

FIGURE 7

Covalent Coupling of Peptides to Microtiter Plates

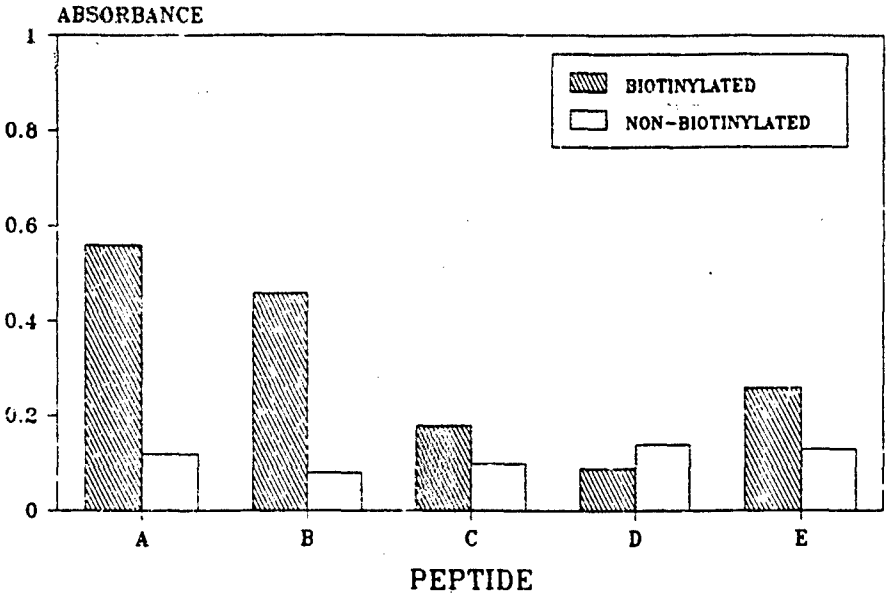
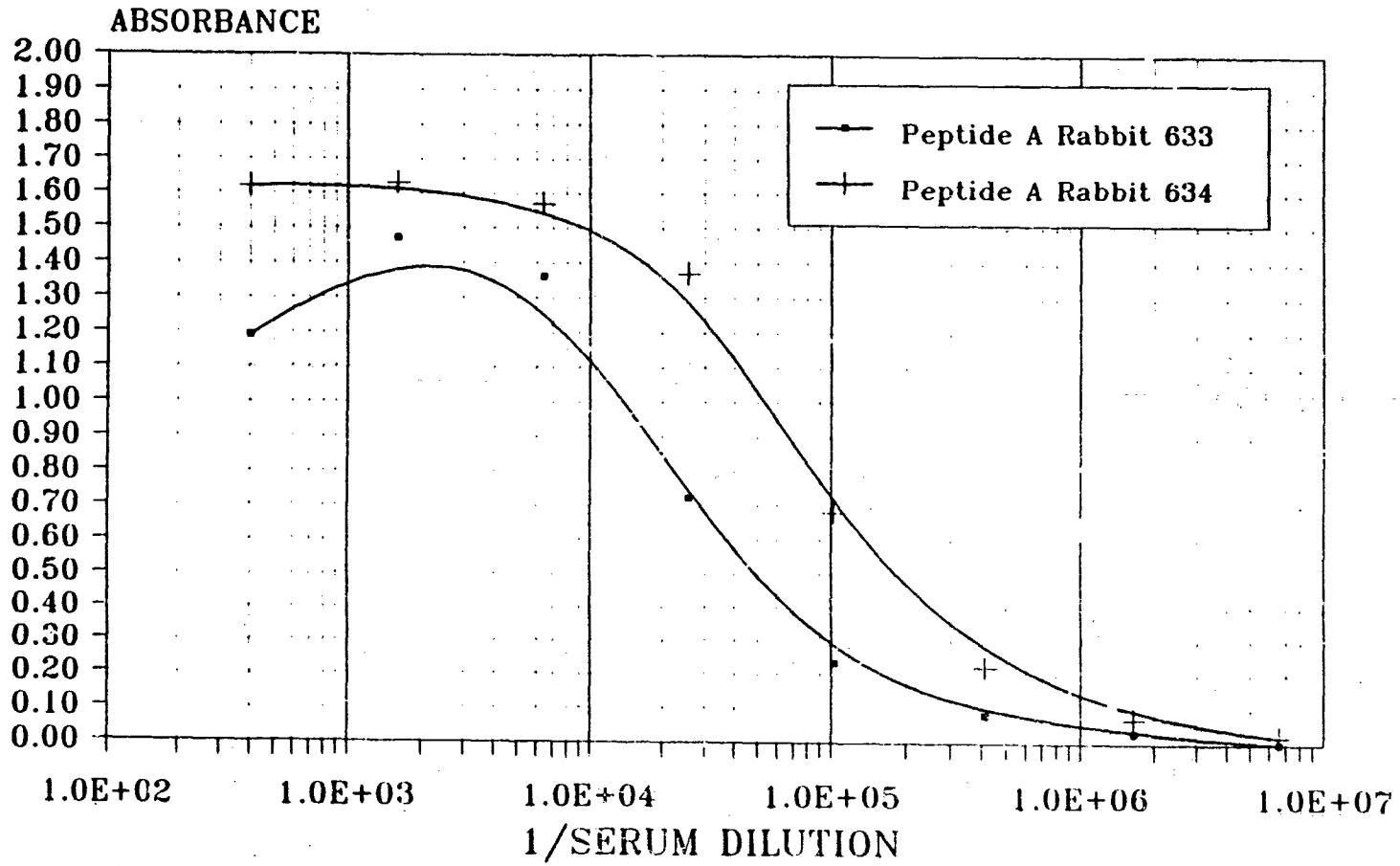


FIGURE 8

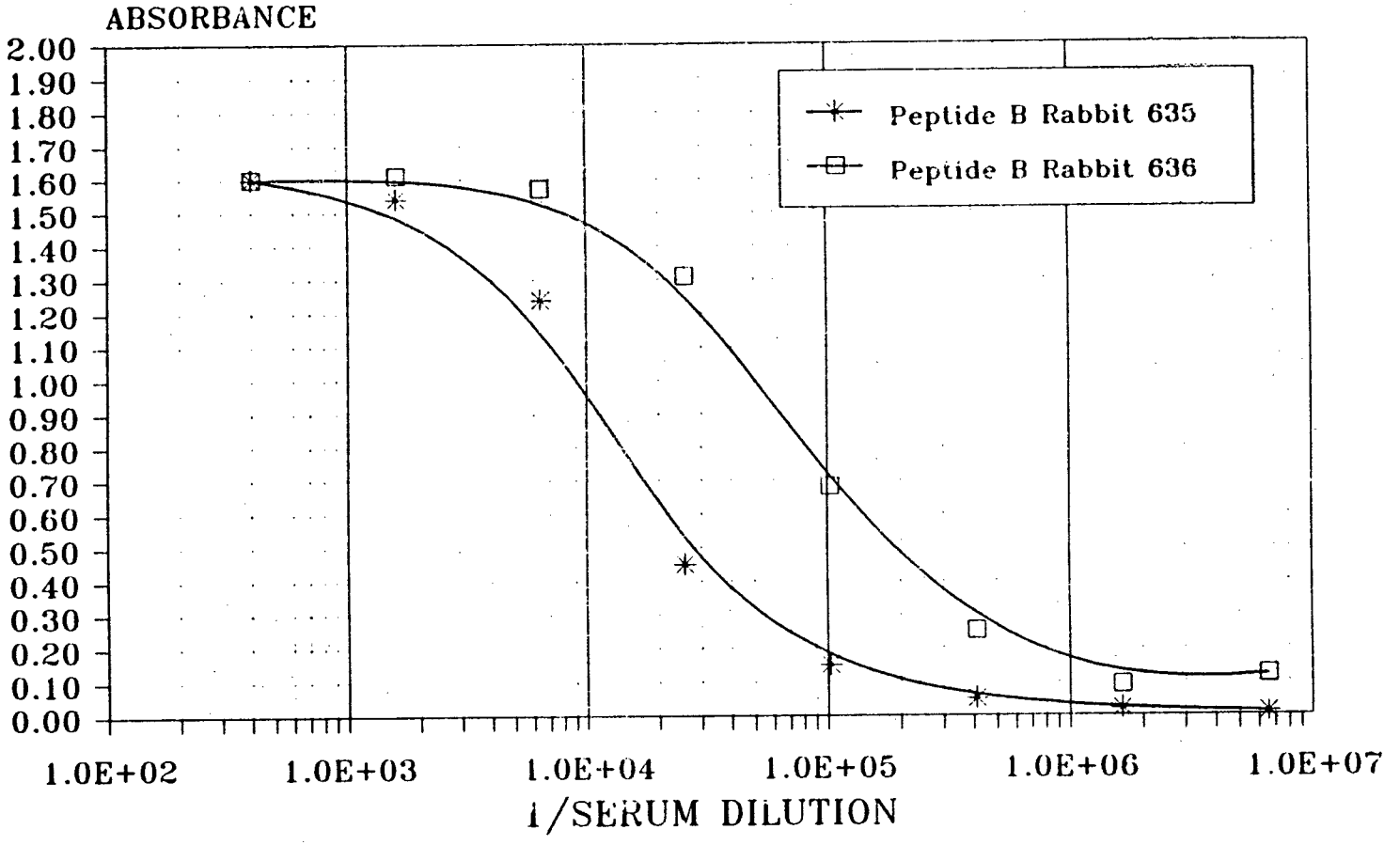
Antibody Titer of Peptide Antisera on Covalently Immobilized Peptide Plates (Thirty Days After Immunization)



3#BMA-03

FIGURE 9

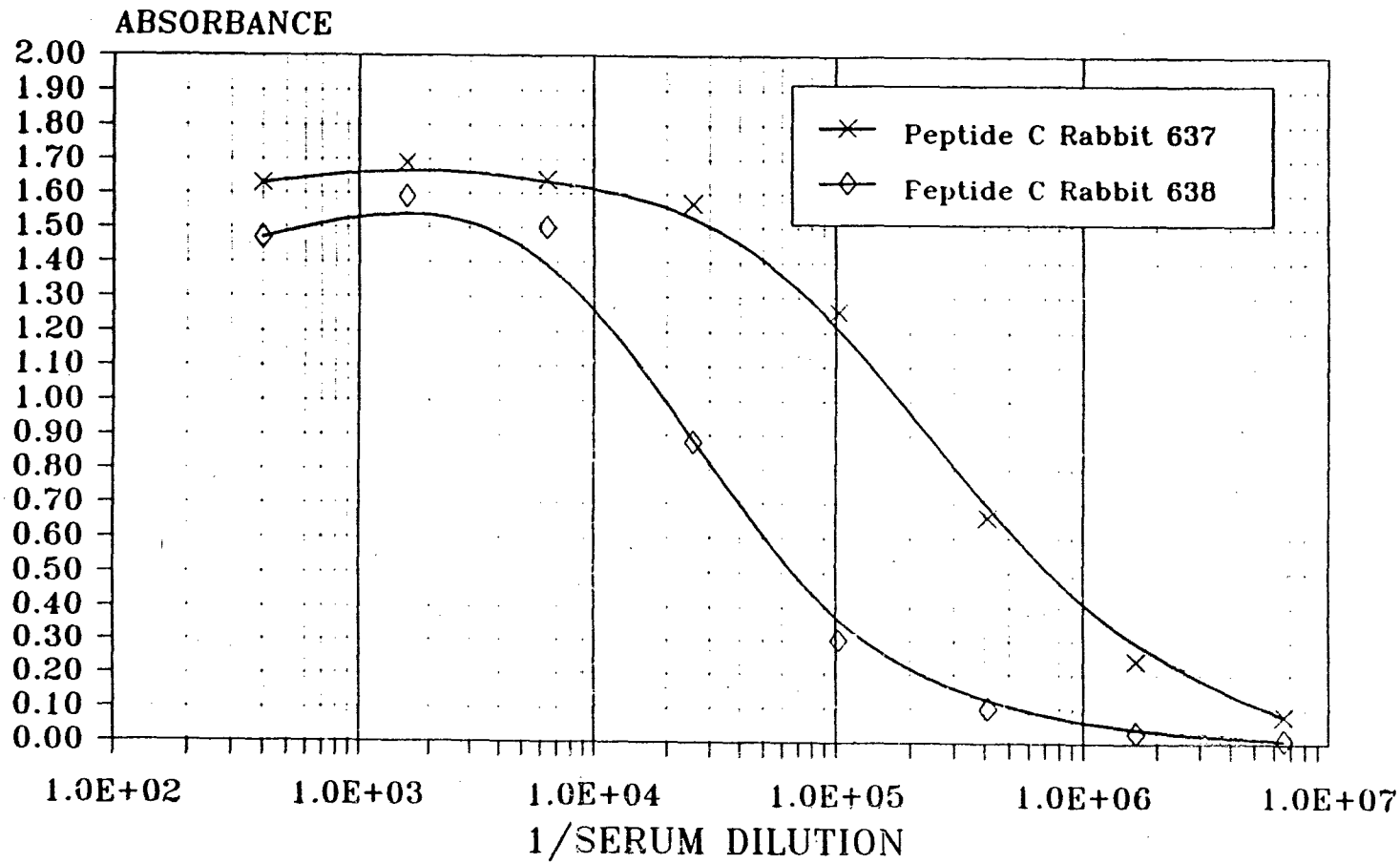
Antibody Titer of Peptide Antisera on Covalently Immobilized Peptide Plates (Thirty Days After Immunization)



3#BMA-03

FIGURE 10

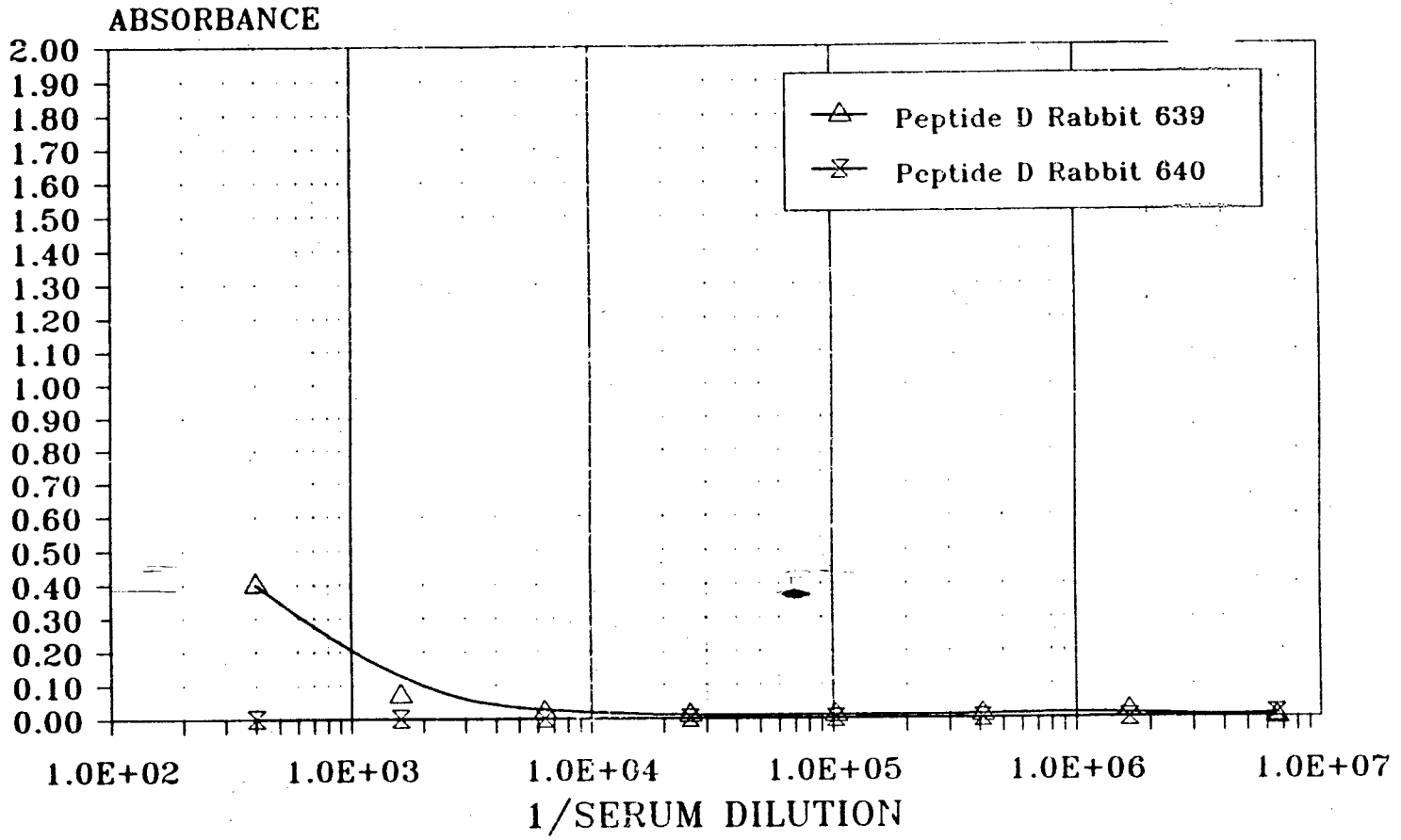
Antibody Titer of Peptide Antisera on Covalently Immobilized Peptide Plates (Thirty Days After Immunization)



3#BMA-03

FIGURE 11

Antibody Titer of Peptide Antisera on Covalently Immobilized Peptide Plates (Thirty Days After Immunization)



3#BMA-03

FIGURE 12

Antibody Titer of Peptide Antisera
on Covalently Immobilized Peptide Plates
(Thirty Days After Immunization)

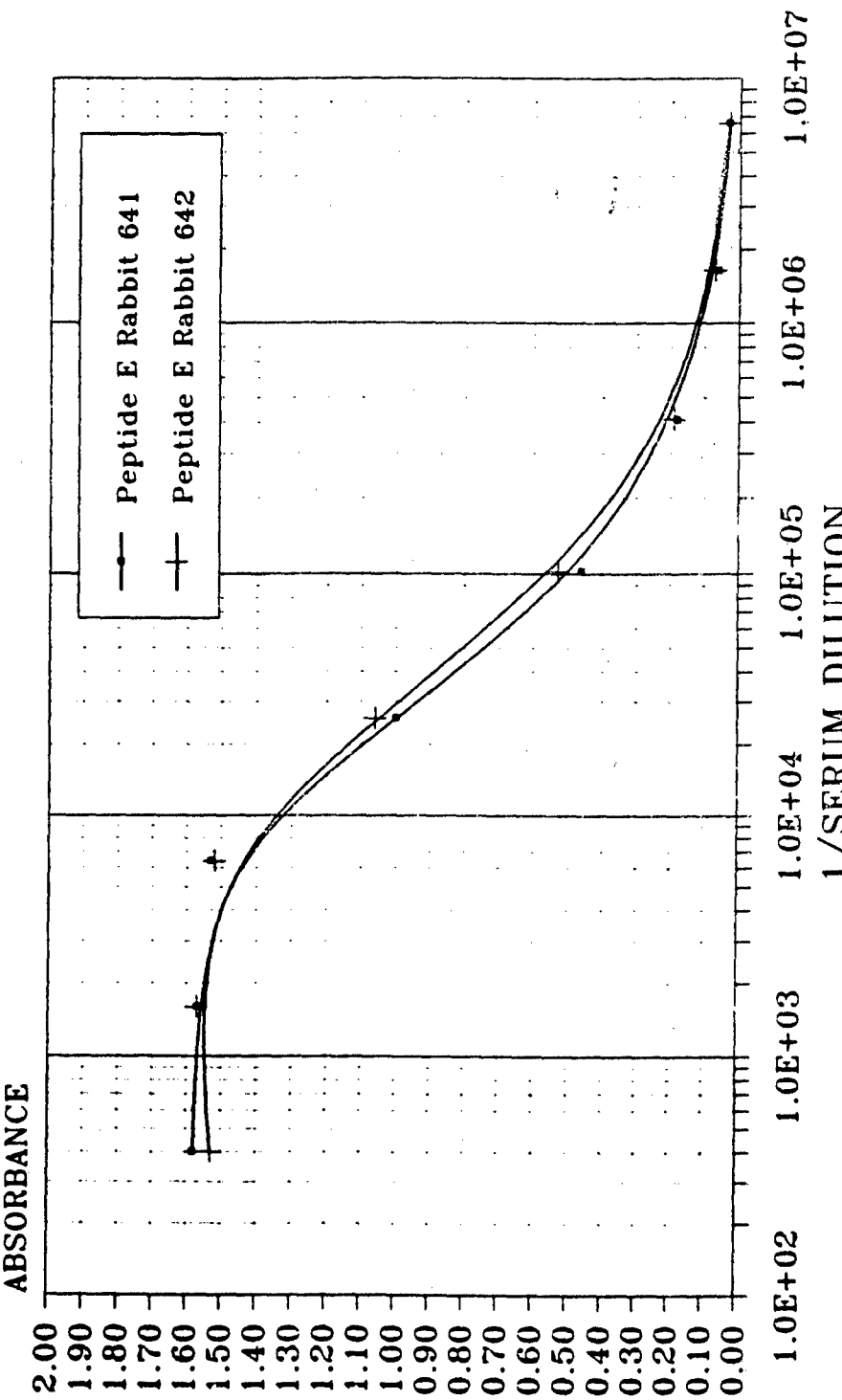
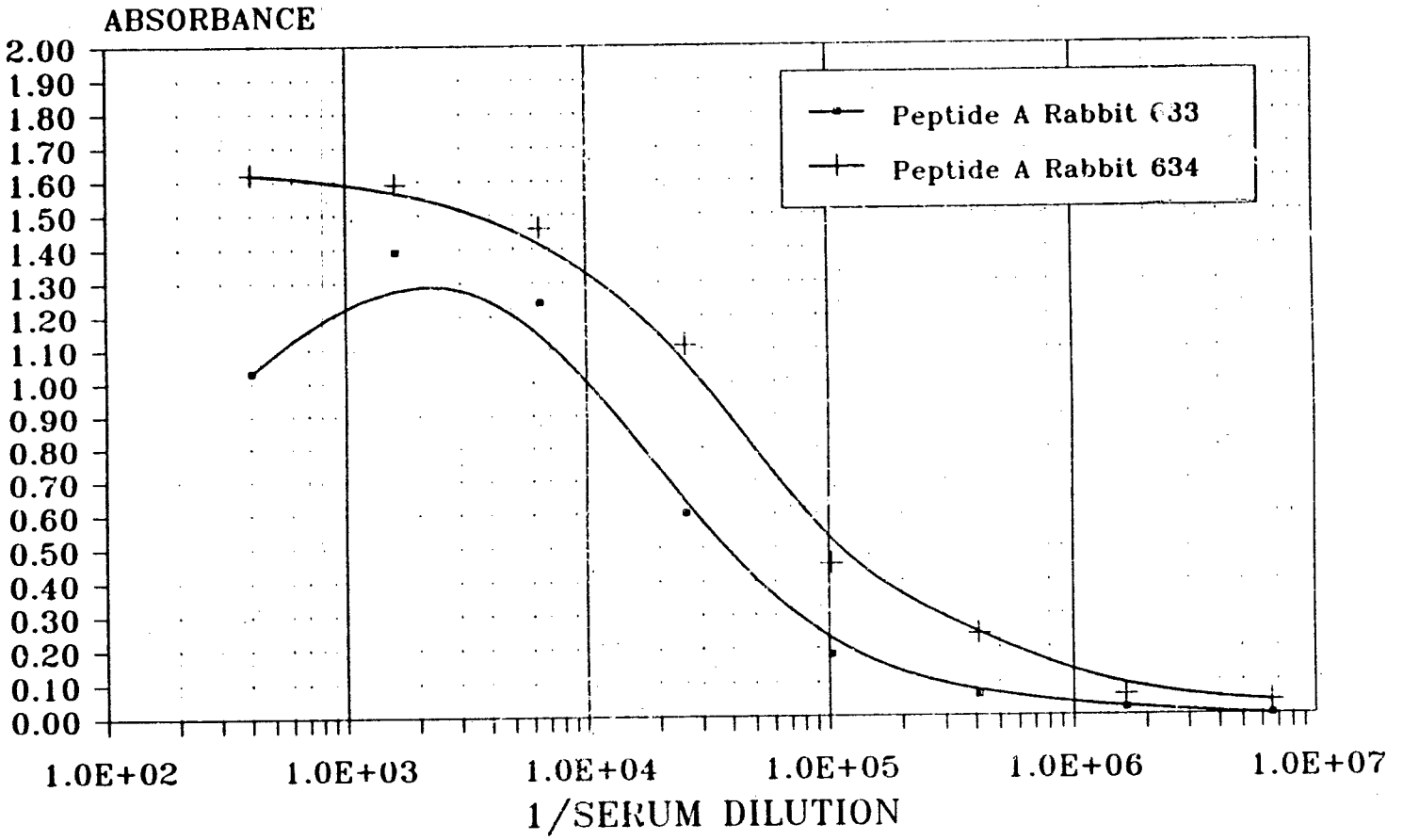


FIGURE 13

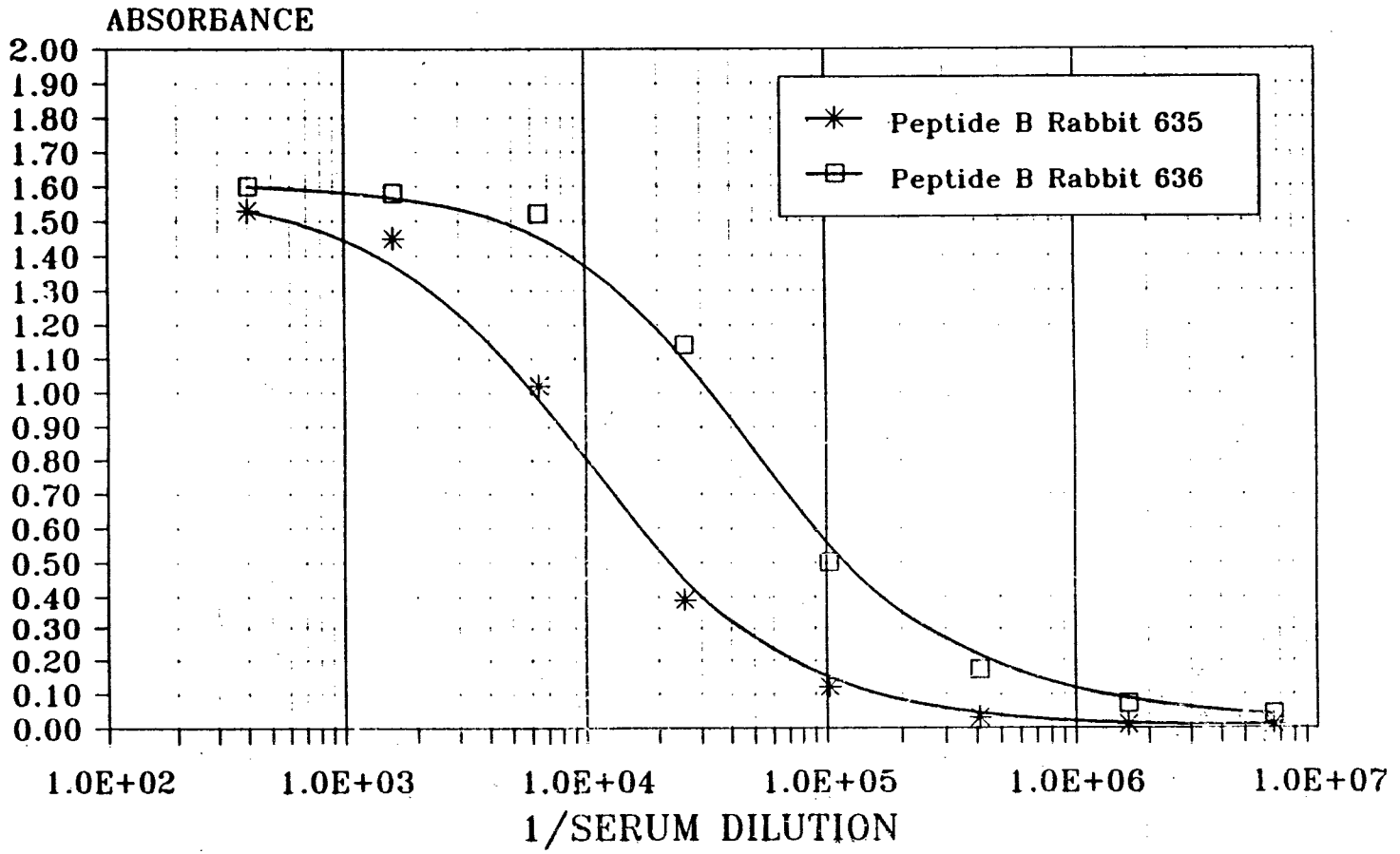
Antibody Titer of Peptide Antisera on Passively Immobilized Peptide Plates (Thirty Days After Immunization)



3#BMA-05

FIGURE 14

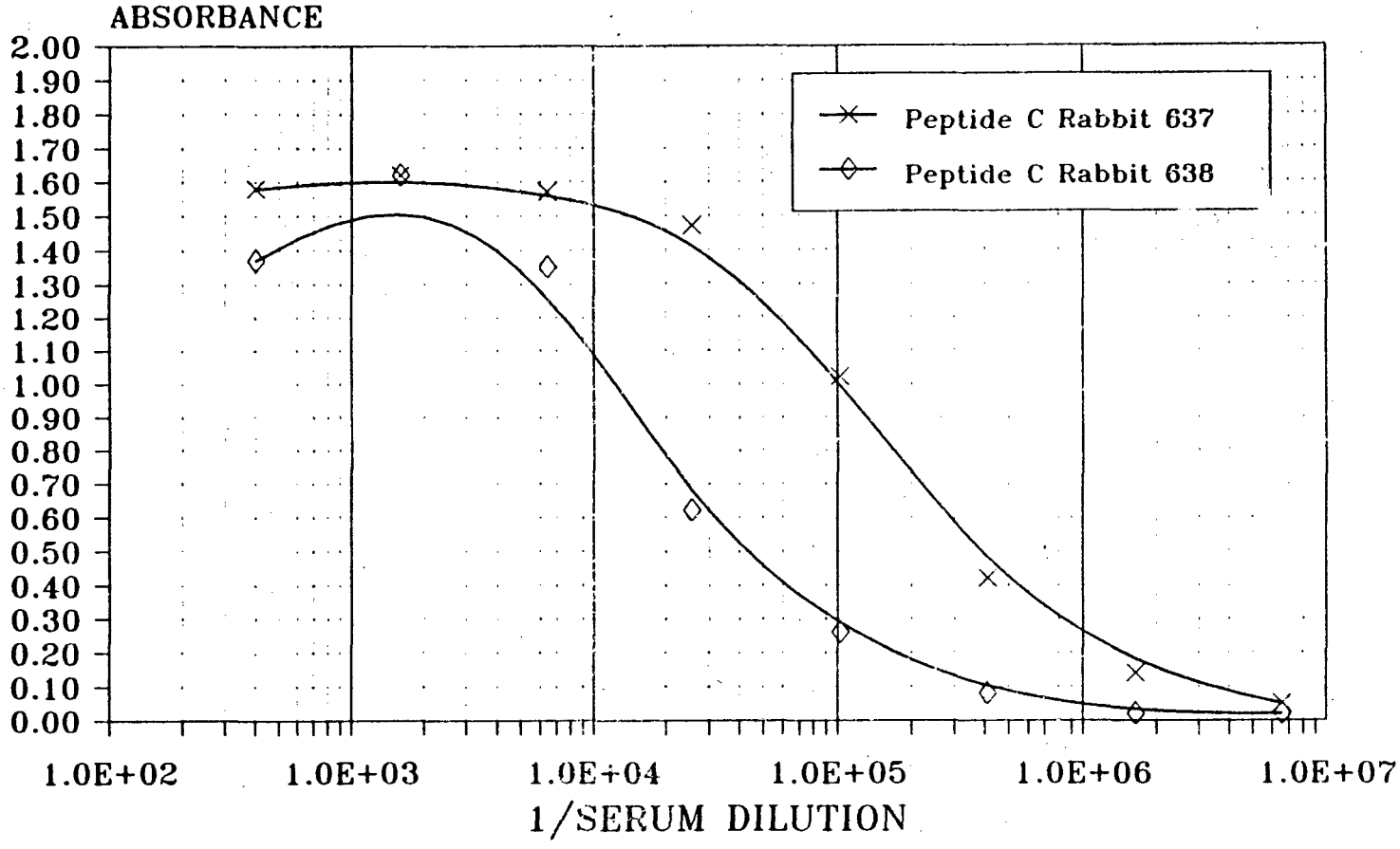
Antibody Titer of Peptide Antisera on Passively Immobilized Peptide Plates (Thirty Days After Immunization)



3#BMA-05

FIGURE 15

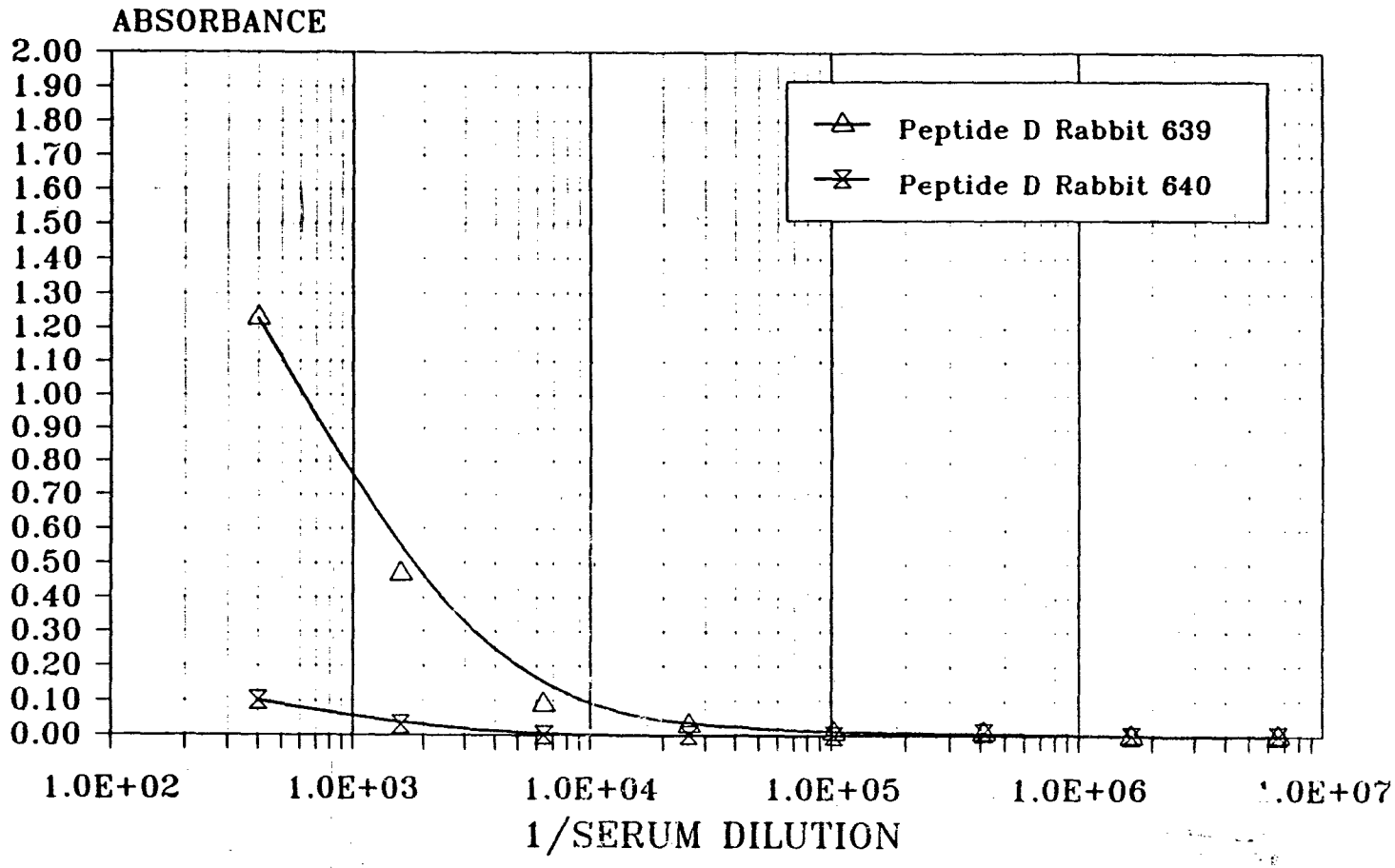
Antibody Titer of Peptide Antisera on Passively Immobilized Peptide Plates (Thirty Days After Immunization)



3#BMA-05

FIGURE 16

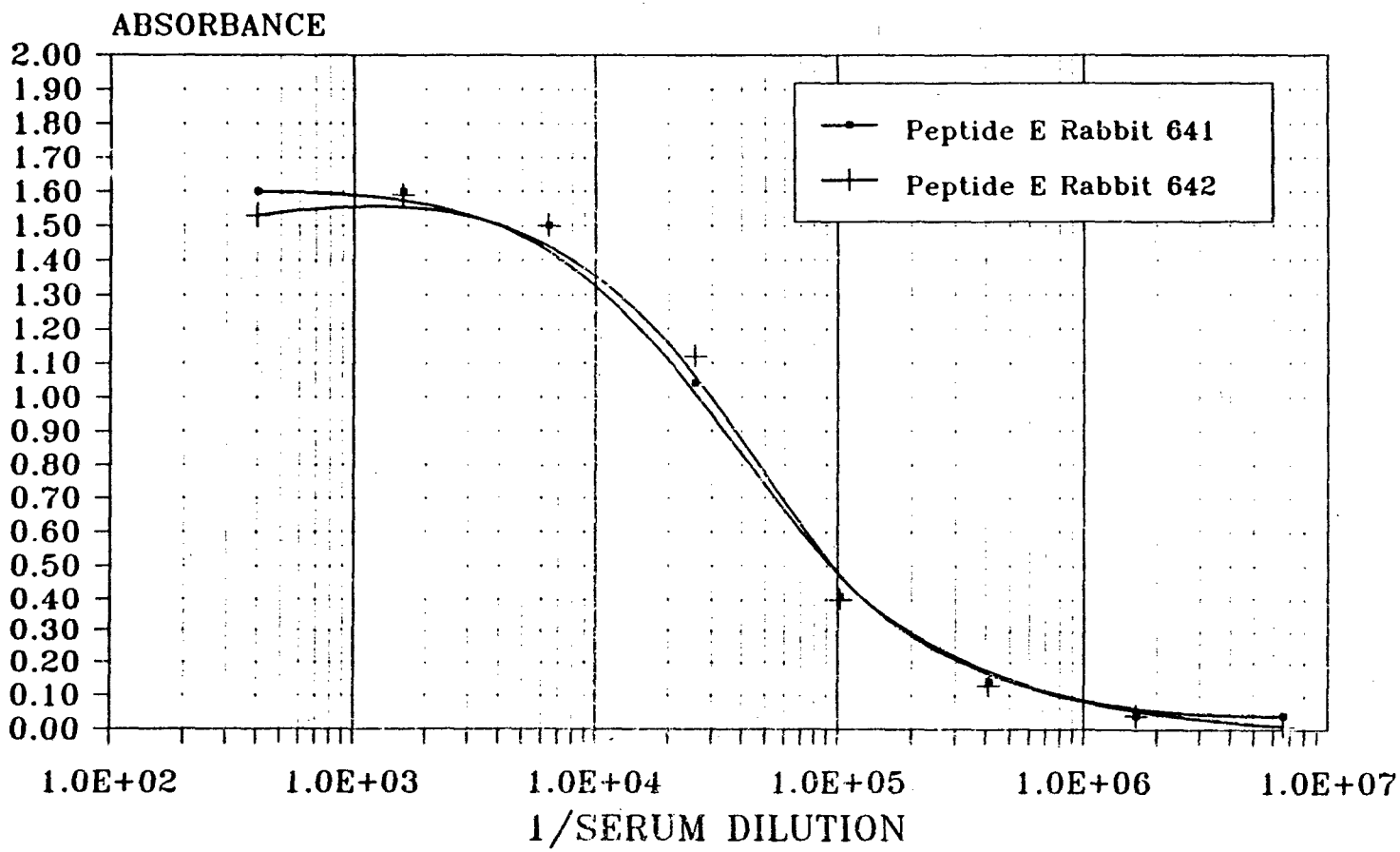
Antibody Titer of Peptide Antisera on Passively Immobilized Peptide Plates (Thirty Days After Immunization)



3#BMA-05

FIGURE 17

Antibody Titer of Peptide Antisera on Passively Immobilized Peptide Plates (Thirty Days After Immunization)



3#BMA-06